SIGNIFICANCE

Physical methods are poised to transform drug discovery and chemical biology by enabling true molecular design. While modeling work is already used extensively in drug discovery, its main role at present is to aid with idea generation or to filter large libraries of compounds for screening. Instead, we imagine using computational techniques extensively to guide the design process. Consider a medicinal chemist in the not-too-distant future who has just finished synthesizing several new derivatives of an existing inhibitor as potential drug leads targeting a particular biomolecule, and has obtained binding affinity or potency data against the desired biomolecular target. Before leaving work, he or she generates ideas for perhaps 100 new compounds which could be synthesized next, then sets a computer to work overnight prioritizing them. By morning, the compounds have all been prioritized based on reliable predictions of their affinity for the desired target, selectivity against alternative targets which should be avoided, solubility, and membrane permeability. The chemist then looks through the predicted properties for the top few compounds and selects the next ones for synthesis. If synthesizing and testing each compound takes several days, this workflow compresses roughly a year's work into a few days.

While this workflow is not yet a reality, huge strides have been made in this direction, with calculated binding affinity predictions now showing real promise [1–8], solubility predictions beginning to come online [9–11], and predicted drug resistance/selectivity also apparently tractable [12], with some headway apparent on membrane permeability [13, 14]. A considerable amount of science and engineering still remains to make this vision a reality, but, given recent progress, the question now seems more one of *when* rather than *whether*.

Recent progress in computational power, especially the widespread availability of graphics processing units (GPUs) and advances in automation [15] and sampling protocols, have helped simulation-based techniques reach the point where they now appear to have sufficient accuracy to be genuinely useful in guiding pharmaceutical drug discovery at least for a certain subset of problems [4–8, 16, 17]. Specifically, in some situations, free energy calculations appear to be capable of achieving RMS errors in the 1-2 kcal/mol range with current force fields, even in prospective applications. As a consequence, pharmaceutical companies are beginning to use these methods in discovery projects.

Unfortunately, these methods still have severe limitations, and a great deal of science and engineering is still needed before these techniques can achieve the major design impact we desire. For example, even "small" protein conformational conformational changes can yield errors up to 5 kcal/mol in calculated binding free energies [18], and force field limitations can pose major challenges as well [19]. Worse, the most important sources of error are not always clear.

Unfortunately, neither retrospective tests nor application of these techniques in drug discovery provides the necessary impetus and data to rapidly advance physical modeling techniques. While large-scale retrospective tests can be valuable for assessing how well we can currently do *retrospectively*, they are of limited value for paving the way forward or for assessing how well we will do for molecular design. Partly, retrospective tests allow for unintentional cheating, where a well-meaning researcher might apply a protocol they know will work in this particular situation where application in a design setting would lead to a different choice of protocol. For example, if the binding mode of a ligand is already known crystallographically, a researcher may use that binding mode in retrospective tests, whereas prospective or design work would require also selecting candidate binding modes, introducing substantial additional uncertainty [1, 20, 21]. This also means that in retrospective tests, researchers almost invariably try far fewer methods than in prospective tests, resulting in much less new insight. Prospective application, while important, also does not provide the necessary impetus, partly because often, the predicted compounds are in fact never tested [6] or the experimental data necessary to assess the quality of the predictions is absent – for example, because binding affinities are not measured or no crystallography is available.

To rapidly advance these methods, we need a series of community blind prediction challenges focused specifically on pushing the limits of predictive techniques, beginning from problems which are just barely tractable with today's methods and advancing to problems just past the frontier. These challenges should be designed to have precisely the necessary high quality experimental data, but also be prospective, predictive tests. While the Drug Design Data Resource (D3R [22], discussed further below) provides an existing community blind challenge on protein-ligand binding, it focuses on using *existing* pharmaceutical datasets for blind challenges, and not on introducing new data in a carefully controlled manner in order to maximize the learning value to the community [22]. In other words, D3R serves well to assess where we are now – but we need a carefully designed effort that will help the field achieve our goals.

One series of blind challenges, called "Statistical Assessment of Modeling of Proteins and Ligands" (SAMPL) provides a model we carry forward and extend here. SAMPL was begun by OpenEye software in 2007/2008 [23] at one of their CUP science events, and has run approximately every two years since then [24–31]; it transitioned to

being run by an outside group of academics beginning partially with SAMPL3 in 2012, then completely for SAMPL4 (2014) and SAMPL5 (2016), with the PI of this proposal playing a key organizational role in SAMPL3-SAMPL5. SAMPL is modeled as a *challenge* rather than a *competition*, with a key goal being to maximize what is learned rather than to declare winners and losers, with the idea that learning will provide the greatest long-term rewards in terms of progress in the field. SAMPL has always involved a component focused on calculation of relatively straightforward physical properties such as hydration free energies, but also introduced host-guest systems as model binding systems for SAMPL3-5, supplementing protein-ligand challenges (on trypsin and HIV integrase) which appeared in SAMPL3-4. SAMPL has already been a tremendous resource for the community, resulting in roughly 100 publications (some are coming out as of this writing) which are typically cited 5-50 times or more each [refs].

Here, we continue the legacy of SAMPL, but design a new series of SAMPL challenges specifically to maximize learning value to the community. Until now, this has been impossible, because SAMPL has been entirely unfunded, so its existence has required "donation" of data from various sources rather than data collected specifically to drive improvements to modeling. Funding of SAMPL will also allow SAMPL to deliberately bridge the gap between calculations of simple physical properties like hydration – which can be calculated fairly accurately with today's methods [28] – and the D3R Grand Challenges on protein-ligand binding, which are a major source of consternation for the community so far [**?**, **?**, **?**, 22]. Unless this gap is bridged, there is the very real possibility that modeling may simply continue to fall far short of expectations in pharmaceutical challenges like D3R for reasons which are unclear. The extension of SAMPL proposed here will allow us to form blind challenges designed to highlight major reasons for failure and drive progress towards resolving them.

Our major goal is to rapidly advance predictive modeling to where it can guide experimental work doing biomolecular design, and extension of the SAMPL challenges will do exactly that. The Computer Aided Structure Prediction (CASP) series of competitions provide an example of how a focused effort can realistically have a dramatic impact on predictive modeling in a relatively short space of time. **[JDC to write text here]** While our model for the SAMPL challenges is slightly different than that for CASP (for example, we have less emphasis on competition) we see SAMPL playing the same key role in driving predictive modeling, but in our field of protein-ligand interactions. In our view it will play a vital role in enhancing the work being done on *existing* data by D3R.

INNOVATION

Blind predictive challenges – and SAMPL in particular – already play a key role in fostering innovation in the field, especially in the form of method development, testing, and hardening, as well as force field development. Thus, this work, by continuing and extending SAMPL, will advance innovative new science. It is worth briefly highlighting several historical examples, though far more are available in the SAMPL literature. The first several SAMPL challenges on hydration free energies had rather hit-and-miss performance, highlighting pitfalls of existing methods and force fields which led to marked improvements in PB models [refs], recognition of some limitations of fixed-charge force fields [refs], repair of some of these force field deficiencies via additional polarization or introduction of off-site charges [refs], and helped motivate alternate implicit or hybrid solvent models [ref]. Together these advances led to a marked improvement in accuracy for calculations of hydration free energies between SAMPLX and SAMPLY (Figure []). Shifts in protonation state and tautomer proved particularly important in the recent SAMPL5 $\log D$ challenge[refs], as they presumably will be for protein-ligand binding as well. Host-guest binding studies have also been particularly important[ref benchmark sets], highlighting the importance of salt effects [refs] and in some cases revealing more severe force field limitations than observed in hydration and distribution challenges [ref Gilson group stuff], pointing the way forward for improving predictive models of molecular interactions [ref].

This work is also innovative because of the uniqueness of SAMPL. While there are other predictive challenges in the area of biomolecular modeling, such as D3R [ref], the pKa cooperative [ref], and CASP [ref], none are specifically focused on driving quantitatively predictive protein-ligand modeling. SAMPL is unique because – at least with the deliberate design plan we propose here – it will be specifically tailored in order to drive improvements in modeling for biomolecular design. It also plays an enabling role for a wide variety of other science, rather than functioning as a stand-alone entity. SAMPL benefits the whole modeling community – for example, even docking software has improved from SAMPL hydration challenges [ref Coleman], and even commercial software has introduced new features and made improvements based on SAMPL challenges [cite Klamt stuff]. In effect, SAMPL serves as an innovation engine, and this work will ensure that SAMPL not only continues but becomes even more valuable to the community.

This work also focuses on innovative experimental methods. Specifically, in Aim 3, we are developing new, high-throughput experiments for studying and measuring protein ligand binding, with careful automated error analysis,

and a heavy emphasis on robotics and automation. The Chodera lab – responsible for Aim 3 – already has substantial expertise in the area of automation of experiments and better uncertainty analysis of those experiments. Additionally, they design their own 3D printed parts to facilitate these experiments, and make plans for these available to others. Thus this work is at the forefront of innovation in high-throughput, automated experiments to produce high quality data with well characterized uncertainties. Not only will the data itself be of prime importance to the community, but the techniques themselves will help future experimental work.

In Aim 4, we will not only run SAMPL community challenges, but also perform our own reference calculations with the latest techniques, testing their accuracy and using these to assess the current state-of-the-art. Both the Mobley and Chodera labs are experts in development of free energy methods for application to physical properties (e.g.. [a couple refs]) and binding (e.g. [a couple refs]), and the reference calculations we perform in Aim 4 are particularly important for innovation as well, serving several key roles: (1) Benchmarking our latest method developments against current "best practices" methods (by doing calculations via both approaches); (2) Facilitating learning, allowing others to experiment with how a change in method or force field impacts results; (3) Focusing the field on key issues by doing sensitivity analysis to whether conditions such as ionic strength, protonation state, tautomer choice, etc. impact computed values.

Innovation in Aim 4 extends beyond reference calculations to analysis of the challenge itself. When methods differ in performance, it is critical to understand whether the differences are statistically significant and important, and to provide an accurate accounting of the uncertainty in performance measures. Thus, careful and innovative analysis of challenge outcomes is particularly important in SAMPL [refs], in some cases driving experimentation with new performance metrics [refs]. Additionally, we try to draw attention to and promote analysis of model uncertainty (as distinct from statistical uncertainty) in calculated values [refs], as understanding the confidence levels of predictions is particularly important for guiding molecular design.

APPROACH

Our approach to systematically advancing modeling for biomolecular design involves collecting carefully tailored experimental data for challenges focusing on physical property prediction, host-guest binding, and biomolecular binding. Thus, we have four main aims, three of which focus on tailoring and generating this experimental data, and a fourth which focuses on running SAMPL challenges to advance modeling and maximize the learning value to the community. These SAMPL challenges will run yearly (though not necessarily every aspect of the proposal will feature in every SAMPL challenge), so each of the aims focused on generating experimental data has a series of stages which correspond to individual future SAMPL challenges.

Aim 1: Generate new data for "simple" SAMPL blind challenges on physical property prediction. Our first aim focuses on generating solution-phase physical property data for small, drug-like molecules – essentially continuing the tradition begun with hydration free energies in SAMPL0-4 and continued with water-cyclohexane distribution coefficients in SAMPL5. Distribution coefficients proved tremendously valuable to the community in SAMPL5 for a number of reasons, highlighting a number of key issues where modeling needs to improve, so they will form the basis for the physical property component in SAMPL6 and return in several subsequent SAMPL challenges.

Distribution coefficients proved to be precisely the right level of difficulty for a SAMPL challenge focused on maximizing learning. Specifically, they were challenging enough that many methods performed poorly, with even the best methods having accuracies less than would have been expected based on their ability to calculate hydration free energies in water [32]. At the same time, methods typically did well enough that it was possible to learn a great deal from examining failure, and the major sources of error were issues which will also plague prediction of ligand-receptor interactions. These included neglect of changes in protonation state on transfer between environments, uncertainty as to the relevant protonation state and/or tautomer in one or both environments [32], problems with sampling the conformation of some of the larger ligands [32, 33], and force field limitations [34]. Our partnership with Genentech for these measurements also meant that the compounds were from Genentech's library and thus very drug-like, unlike typical compounds seen in hydration free energy challenges of the past. In some respects, distribution coefficients posed the ideal SAMPL challenge, hitting the sweet spot in terms of difficulty – difficult enough that clear failures were frequent and there is much room for improvement, but not so difficult that the reasons for failure were unclear in general. Still, the challenge could have been improved by follow-up experiments to re-check some of the experimental results [30, 34–36] – but without funding for someone to continue working in this space, this has so far been impossible.

Because distribution coefficients were so valuable in SAMPL5 and are driving so much needed method development [refs], we will measure new cyclohexane-water distribution coefficients for our first data set here and this will form the basis for the physical property component of SAMPL6. Because octanol-water distribution coefficients are also

potentially tractable [32], and measured much more frequently experimentally, we plan to generate measurements for the same compound distributing between both cyclohexane-water and octanol-water for this challenge.

However, distribution coefficients conflate several issues which are still complex, namely protonation state and tautomer prediction, as well as transfer into different environments. Thus, we will likely need to turn to separating these issues to improve our handling of them one at a time. For SAMPL7, then, our tentative plan is to measure pKa values for an extensive set of drug-like molecules in water and provide data specifically on pKa values, thereby separating the issues of predicting protonation state from those of transfer.

In the next challenge, SAMPL8, we would re-combine the pKa and transfer issues in a way to maximize learning – specifically, we will not only measure distribution coefficients but also measure pKa values for the same set of compounds, allowing participants to (a) predict distribution; (b) predict pKa; and (c) predict partitioning.

Several other avenues are of interest for future datasets as well. New computational techniques are targeting membrane permeability [13, 14], and this is experimentally accessible (see support letters from Pfizer and Merck), leading to potential interest in new datasets and challenges focused there. Alternatively, partition/distribution into other environments aside from cyclohexane or octanol may provide significant value, especially given dielectric constant issues posed by current force fields [ref].

Here, our experimental work will be performed with partners in the pharmaceutical industry, following roughly the model used for SAMPL5, where we partnered with Genentech, sending Bas Rustenburg, a graduate student in the Chodera lab, to conduct measurements of $\log D$ using existing equipment. We find that pharma often has substantial access to equipment – such as the expensive Sirius T3 for measuring partition, distribution, and pKa – which is very hard to find in academia, and it may not always see heavy use. Thus, sending a person into pharma to generate data can be an extremely fruitful line of inquiry, as the relevant equipment and expertise is already present but manpower is lacking. Here, we are partnering with Genentech, Pfizer, and Merck (see support letters), who have agreed to give us access to the equipment and expertise we need for the necessary measurements. This work will provide funding for us to send a joint experiment and modeling student from the Mobley group to conduct the relevant measurements with our pharma partners. As noted, we already know that this model will work given our experience in SAMPL5 [36], and our pharma partners see the value of this data and the SAMPL challenge to the modeling community.

Aim 2: Measure binding of novel host-guest complexes for introductory ligand binding challenges. We will measure new host-guest binding free energies for cucurbiturils and deep-cavity cavitands, yielding further host-guest binding challenges which span between physical property prediction and protein-ligand binding. Host guest systems are some of the simplest cases of molecular recognition, and thus these binding data will drive improvements in modeling of simple binding systems with techniques of relevance to drug discovery.

Cucubituril-based receptors as model binding systems

Cucubituril derivatives for host-guest binding. The Isaacs group has previously participated in the SAMPL challenges and supplied unpublished host-guest binding constants [**?**, 37, 38]. Our participation was quite stimulating for us and influenced our investigation of the biomedical applications of acyclic CB[n]-type receptors (a.k.a Calabadions). Cucurbit[n]uril receptors are particularly well suited for the SAMPL challenges because they exhibit: 1) high binding constants toward suitable guests in water (routinely μM to nM; occasionally pM to fM) [39–45], 2) high selectivities between structurally related guests which translate into large $\Delta\Delta G$ values [46], 3) low molecular weights (1000-2000 amu) which allows high levels of theory to be used, and 4) limited conformational degrees of freedom. Herein, we propose to continue to participate in the next three SAMPL challenges during the proposed five year funding period by resynthesizing previously published CB[n]-type receptors of increasing complexity, measure Ka values and determine host-guest stoichiometry and geometry toward biologically relevant guests which will allow the computational chemists to push the boundaries of the free-energy prediction of receptor-igand complexes. Figure 1 shows the chemical structures of three hosts – Me4CB[8] [47], glycoluril hexamer [48], and acyclic CB[n]-type receptors [49–54] which span the range from preorganized macrocyclic host to uncharged acyclic but preorganized host to highly charged acyclic host.

SAMPL6. For this challenge we propose to measure K_a and ΔH values, stoichiometry, and geometry for the interaction of Me4CB[8] (nicely water soluble CB[8] derivative) toward 15 guests (chosen from top selling drugs, Table CB1) by either direct or competition isothermal titration calorimetry (ITC), UV/Vis or fluorescence indicator displacement assay, or NMR competition experiments which we are very experienced with [39, 40, 55, 56]. Our selection of Me_4CB[8] and top 100 drugs was based on a desire to increase the level of complexity of the computational challenge by: 1) changing host flexibility (e.g. Me_4CB[8] can exhibit ellipsoidal deformation) [47],

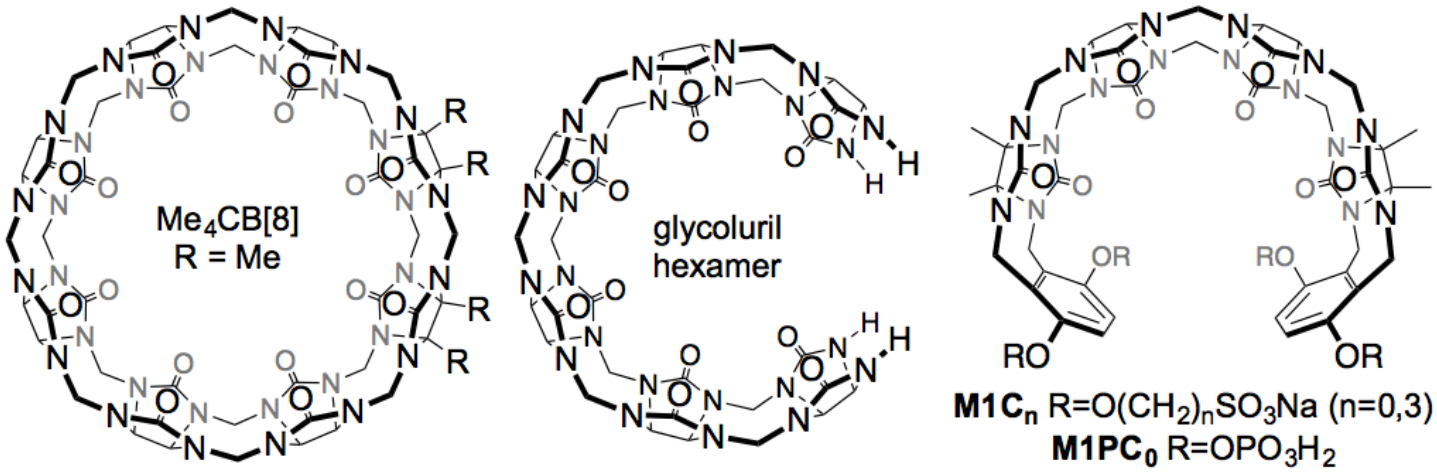

Figure 1: **Structures of Me_4CB[8], glycouril hexamer, and acyclic CB[n]-type receptors.**

2) by allowing the possibility of binary or ternary (e.g. 1:1 and/or 1:2 host:guest) complexes [57–59], 3) using drugs with several potential binding epitopes to include sampling issues. Host:guest stoichiometry and geometry (e.g. which binding epitope is complexed) will be addressed by ITC "n" values, Job plots monitored by UV/Vis or NMR [60], and by 1H NMR complexation induced changes in chemical shifts [61]. All three sets of studies will be conducted in phosphate buffered saline (pH 7.4 with physiological salt) which introduces further complexity due to competitive interaction between the C=O portals of CB[n]-type receptors and metal ions via ion-dipole interactions which reduces the observed Ka values [62].

drug	**features**
memantine	adamantane; 1:1
saxagliptin	adamantane; 1:1
premarin	steroid
pancuronium	steroid
varenicline	1:1 vs 1:2
valsartan	pKa 4.37
omeprazole	pKa 4.77
ranolazine	pKa 7.17; epitopes
pradaxa	pKa 3.87; epitopes
nilotinib	epitopes; pKa 6.3
sensipar	epitopes; folding
vyvance	diamine; epitopes; folding
minocycline	tetracyclin; amino aniline

Table 1: Selected drugs as guests

SAMPL8. We propose to study host:guest complexes of glycoluril hexamer toward the 15 drugs (Figure CB1). We select glycoluril hexamer for this challenge because it: 1) increases the conformational dynamics of the host, and 2) influences the number and energy of solvating (and unusually coordinated) water molecules that are implicated in the observed high binding constants for CB[n]-guest complexes [45, 63]. Furthermore, in selecting the drugs, we have chosen several that have pKa values in the 3.8 to 7.4 range. Similar to biomolecular host-guest systems, CB[n]-type receptors are well known to induce pKa shifts (up to 4 pKa units) of complexed guests [64–66], and the ability of computation to replicate and predict such shifts and their impact on Ka are of high significance.

SAMPL10. We will focus on acyclic CB[n]-type receptors (e.g. $M1C_3$, $M1C_0$, and $M1PC_0$ that contain anionic solubilizing groups attached via different linker lengths. As in SAMPL2, these acyclic CB[n]-type receptor introduces conformational complexity and influences the free energy of the solvating H_2O molecules in the free host. Moreover, the presence of 4 anionic groups in close proximity to the cavity are expected to have a significant influence on the balance between ion-dipole interactions and the solvation of the free host.

Gibb deep cavity cavitands for host-guest studies

History of octa-acid SAMPL challenges. During SAMPL4 [67] and SAMPL5 [68] we focused on two hosts: the octa-acid 1 (R = H) and another octa-acid derivative with four methyl groups positioned at the portal of the binding pocket (1, R = Me). These studies used Isothermal Titration Calorimetry (ITC) to measure the thermodynamics of (1) host 1 (R = H) complexing a range of carboxylate guests, and (2) the binding of carboxylate and trimethylammonium guests to both hosts (1, H = H and Me). In both cases ^{1}H-NMR titration was also used in a confirmatory role for ITC-derived free energies of binding. SAMPL5 emphasized how differences in the shape of the hydrophobic pocket of the host can have a profound affect on affinity for some guests.

Novel deep cavity hosts probe the effects of binding site charge constellations. For future SAMPL challenges, we will expand on the range of hosts by including 2 and 3 in our ITC studies. Like cavitand 1, host 2 is an

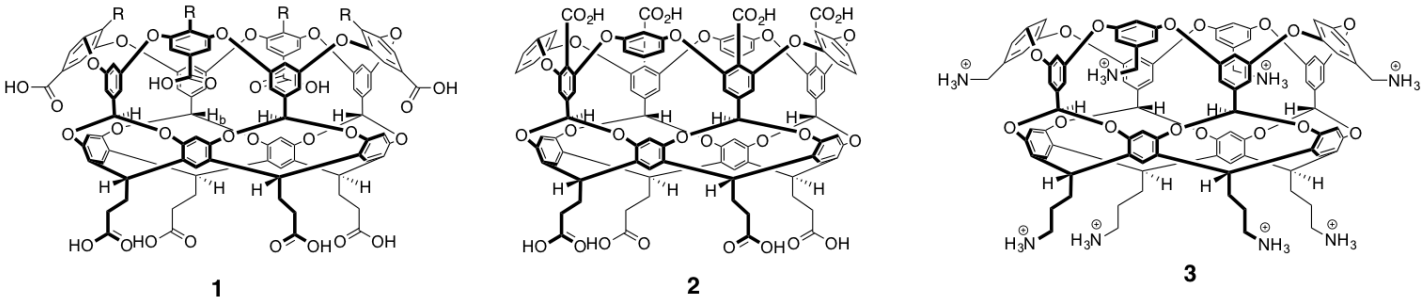

Figure 2: **Gibb deep cavity cavitands for SAMPL6-10.**

octa-acid derivative. However, the four benzoate groups are relocated from the extreme exterior in the case of 1, to the rim of the binding pocket in 2. We surmise that this will have a direct effect on the binding of charged guests, but more subtly, an indirect effect on guest complexation via changes to the solvation of the empty host. Octa-trimethylammonuim cavitand ("positand" 3) has the same overall architecture as host 1, but inverts the charges on the water solubilizing exterior coat. While it is not yet clear if this switch in groups relatively remote from the pocket will directly affect guest complexation, results from related systems suggest it can (unpublished).

Guests for the five proposed ITC studies will be obtained from commercial sources, focusing on molecules that probe the limitations of current force-fields as well as new data as it is gathered.

SAMPL6-10 deep cavity cavitand challenges. The host-guest challenge for SAMPL6 will focus on how well the effect of host carboxylate substituent location can be predicted, and will involve hosts 1 and 2 with a set of five, previously uninvestigated guests. SAMPL7 will provide a second iteration of this experiment to test algorithmic improvements in predictive modeling following SAMPL6 by comparing hosts 1 and 3 with a different set of guests. We anticipate that because of the relative remoteness of the charged groups in these two hosts, the effects of switching charges will be subtler than the differences between 1 and 2. SAMPL8 will consider the effect of common biologically-relevant counterions/salts salts on guest binding, comparing the effects of NaCl and NaI on the complexation of five guests to 1. We have previously shown that iodide has a weak affinity for the binding pocket of 1, whilst sodium ions have an affinity for the outer carboxylates [69], requiring modeling to capture the differential affinities of these ions in addition to guest affinities to successfully model the observed affinities. SAMPL9 will follow up on this by examining the effects of these same two salts on the complexation of five guests to 3, again giving the modeling community time to incorporate algorithmic improvements following SAMPL8. While we have not yet quantified salt affinities to host 3, we expect the iodide to have affinity for both the pocket and the positively charged solubilizing groups. For SAMPL10 we will consider the effects of co-solvents on the binding of five guests to 1 and 2 to probe the effect of co-solvent competition for the binding site, as well as effects cosolvents may have in weakening the hydrophobic effect.

Aim 3. Develop model protein:ligand systems that isolate specific modeling challenges found in more complex pharmacologically relevant systems. A major goal of our effort is to drive advances in the quantitative modeling of protein:ligand interactions. While the Drug Design Data Resource (D3R [22]) effort provides community blind challenges for biomolecular targets of pharmaceutical interest, these targets generally contain a daunting number of complexities that frustrate the ability for current methodologies to achieve quantitative accuracy, resulting in poor performance [CITE]. For example, while kinases are targets of great interest to drug discovery, blind challenges involving kinase targets conflate issues of slow protein conformational dynamics [**?**], protonation state effects of both protein [**?**] and ligand [**?**, **?**], charged ligands, and the modeling of complex divalent salt environments and phosphorylation state effects along with the standard computational challenges of conformational sampling and forcefield accuracy. While the value of these exercises as an accurate prospective benchmark of current-generation model accuracy is unquestionable, **the ability of blind challenges on complex pharmaceutical targets to rapidly advance the field of quantitative predictive modeling is limited.**

Instead, **our philosophy is to identify model protein:ligand systems with the goal of *isolating* individual accuracy-limiting effects in iterative cycles of prospective blind community challenges.** This process focuses the field on identifying and evaluating multiple solutions to the accuracy-limiting effects (such as how to deal with ligand and protein protonation-state issues [**?**], slow protein conformational dynamics, etc.) free from other complicating factors, allowing a direct evaluation of how well the phenomena of interest are modeled. Datasets collected for these blind challenges then become standard benchmark datasets for retrospectively examining the effectiveness of modeling approaches in treating these effects to facilitate comparisons of methodologies in

publications, while future iterations or variations of the same SAMPL experiment allow iterative refinement and prospective blinded evaluations of methodologies. In this way, this cycle of blind challenges utilizing model systems can rapidly drive progress in rapidly overcoming scientific hurdles limiting quantitative accuracy.

While model protein-ligand systems have a long and storied history of driving progress in individual research laboratories (such as the Shoichet T4 lysozyme mutants [**?**]), their power in blind community challenge cycles is amplified by leveraging community participation. An excellent example of this was the collection of a challenge dataset featuring the binding of small, rigid charged molecules to bovine trypsin for SAMPL3 [**?**], which rapidly focused the field on the deficiencies of current alchemical free energy methodologies in treating the binding of charged ligands. Within two years, multiple laboratories had developed and disseminated convergent practical solutions to effectively handle charged ligand binding that are now adopted as best practices [**?**, **?**].

SAMPL6-10 model protein:ligand challenges. For the SAMPL6-10 challenges, we propose to introduce a new model protein:ligand system each year, with challenges fielded for each system for at least two consecutive years to allow iterative methodology improvement and assessment. Immediately following the challenge, challenge data (including all primary data) will be published and released as a version-controlled benchmark dataset for retrospective evaluation. The first challenge (introduced in SAMPL6) will focus on modeling the binding of small soluble drug fragments to a relatively rigid protein with multiple weak binding sites, isolating the ability of current-generation modeling approaches to model weak and multiple binding effects. Because rapidly focusing the field on current challenges in predictive modeling requires the ability to adapt to deficiencies identified D3R/SAMPL challenges of the previous year, subsequent model systems will be rapidly identified and developed using a new informatics platform we have developed to identify tractable model systems.

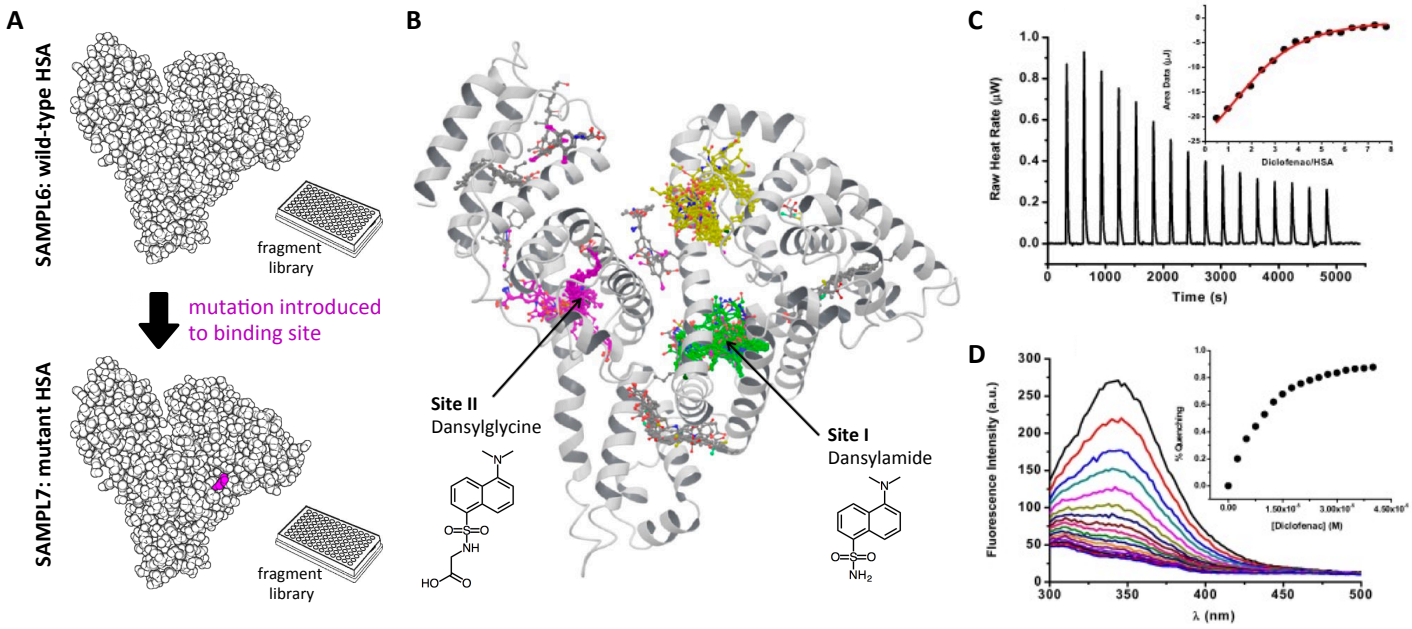

Figure 3. **The SAMPL6/7 protein:ligand challenge focuses on soluble drug fragment binding to human serum albumin (HSA).** *(A)* SAMPL6 will introduce recombinant human serum albumin (HSA) as a target, against which a library of ∼100 small soluble drug fragments will be assayed. By introducing a mutation in one of the binding pockets, we will create a second challenge target for SAMPL7. *(B)* HSA is a relatively stable and inert protein, and is the most abundant protein in blood plasma, with a variety of well-characterized binding sites that have weak affinity for many drugs and drug-like moieties [**?**] (figure from [**?**]). Drug molecules have been observed to bind at up to eight distinct sites, with major characterized sites denoted Site I (green, Sudlow's Site I) and Site II (purple, Sudlow's Site II). Dansylamide and dansylglycine exibit binding-enhanced fluorescence upon binding to HSA, thus a binding curve can be constructed based on increase in fluorescence emission at 480 nm. Dansylamide was shown to bind primarily to Site I (Kd $\sim$ 5 μM) and dansylglycine was shown to bind primarily to Site II (Kd $\sim$ 2 μM) [m2] with *(C)* Binding assay of HSA and Diclofenac measured by isothermal titration calorimetry (ITC). Inset figure is integrated heat vs. Diclofenac/HSA molar ratio of each injection [**?**]. *(D)* Fluorescence emission of HSA changing depending on diclofenac concentration. Inset plot shows binding curve constructed of percent tryptophan quenching (at 346 nm) vs. Diclofenac concentration [**?**] Diclofenac is a fluorescent ligand reported to bind Site II [**?**].

SAMPL6: Assessing predictive modeling to multiple weak binding sites with the binding of small soluble fragments to human serum albumin (HSA). Human serum albumin (HSA), the most abundant blood plasma protein, has the remarkable ability to bind a great variety of small molecule drugs in multiple binding sites (Figure 3B) [**?**]. As a result, HSA is not only an excellent model system for isolating the challenge of binding

multiple weak ligands to a stable rigid protein, it is also a pharmacologically relevant system due to its ability to drastically modulate drug pharmacokinetics [**?**]. HSA has at least *eight* known binding sites, with numerous crystal structures available for drugs binding to two predominant sites (Sudlow Site I and II) [**?**]. Small soluble molecules resembling drug fragments have previously been shown to have a high likelihood of detectable binding to HSA ($\geq$90% of small druglike fragments, as detected by SPR [**?**]), providing an experimentally-tractable diverse set of ligands spanning several orders of magnitude in affinity [CITE]. As current advanced methodologies such as alchemical free energy calculations currently assume a single well-defined binding site with high affinity [**?**], this dataset will allow the isolation of the effect of weak multiple binding from the majority of other counfounding factors in protein-ligand binding.

Recombinant HSA will be expressed in *E. coli* and purified via refolding from inclusion bodies [**?**], and will be defatted at low pH to ensure the resulting protein is free of the complications of both glycosylation and bound fatty acids found in plasma-isolated HSA [**?**]. Recombinant expression will also allow a mutant form of HSA (engineered via quick-change single-primer mutagenesis) to be fielded for a SAMPL7 iteration of this challenge (Figure 3B). We will obtain a diverse library of 96 soluble drug-fragment-like molecules in pre-plated format for which HSA-ligand affinities are not available in the literature as dry compound, and assay them for HSA binding using automated isothermal titration calorimetry (ITC) (Figure 3C), with the goal of characterizing the overall binding affinity of the compound to HSA. The same ligands pre-plated in DMSO format will be used to conduct a separate set of fluorescence competition assays in which the site-specific fluorescent probes daynsylamide (Site I) and dansylglycine (Site II) will be used to measure site-specific affinities for Sites I and II (Figure 3D), allowing participants to validate whether they predicted the correct binding site and, if so, the site-specific affinity.

SAMPL7-10: Rapid, responsive development of new model systems using a novel informatics platform. We have developed a novel informatics platform called TargetExplorer aimed at identifying new protein targets that can be rapidly developed into experimentally- and computationally-tractable model systems focusing on individual challenges. This tool—which will be made accessible to other laboratories via an easy-to-use web interface during the course of this project—successively filters all protein:ligand complexes identified in the PDB according to a list of criteria that allow facile development as a model protein:ligand binding system, as well as identification of systems that isolate individual challenges in modeling accuracy. Experimental tractability includes: (1) the availability of multiple protein:ligand crystal structures; (2) known bacterial expression (e.g. from PDB `EXPRESSION_SYSTEM` records); (3) the capacity to bind a wide dynamic range of ligands (determined via data available in ChEMBL); (4) the availability of multiple known ligands that can be purchased (via ZINC); (5) tractability of experimental affinity measurements, such as known ligands with potentially fluorescent scaffolds (for fluorescence competition assays), highly soluble ligands (for ITC), or ligands above a minimal mass (for SPR or MST). A number of additional filters annotate potential experimentally tractable systems for suitability as a model system that isolates individual challenges, such as: charged ligands or potential ligand protonation state or tautomer [**?**] effects (deduced from predicted aqueous protonation/tautomer energies); potential protein protonation state effects (deduced from MCCE2 calculations [**?**]); protein conformational changes (deduced from variation in protein conformation or the presence of unresolved loops in protein:ligand crystal structures); the presence of post-translational modifications that may affect affinity (deduced from Uniprot annotations); coordinated metals (identified in crystal structures); ordered waters (present in multiple crystal structures); etc.

The Chodera lab has developed an automated wetlab for the purpose of rapidly developing model protein systems using bacterial expression techniques (see Equipment and Facilities). Potential targets matching desired challenge criteria will be screened for bacterial expression using high-throughput cloning, transformation, and expression testing, with purity and yield assessed by capillary electrophoresis on a Caliper GXII. Targets will be screened for stability in various buffers using Thermofluor [**?**]. Ligands identified via the informatics platform to span a wide dynamic range of binding affinities will be purchased as dry powder stocks and prepared for assay by highly accurate gravimetric solution preparation techniques using a Quantos automated balance. A wide variety of biophysical techniques are available to provide accurate, quantitative measurements of protein-ligand binding affinities, including fluorescence (if fluorescent probe ligands are available), absorption (e.g. Soret band shifts), automated isothermal titration calorimetry (provided ligands are sufficiently soluble), surface plasmon resonance, microscale thermophoresis (MST), luminescence, and alphascreen; all except MST are fully automated.

Our approach to developing challenge datasets will be twofold: First, small molecules similar to known ligands will be purchased and assayed, with the presumption that these molecules are likely to have measurable affinities. Second, site-directed mutants will be introduced to modulate the binding affinities of known ligands using single-primer quick-change mutagenesis, which can be performed and screened for expression in 96-well format. Challenge datasets will therefore consist of a matrix of protein mutants and ligands, providing a rich dataset to deeply explore the effects of interest.

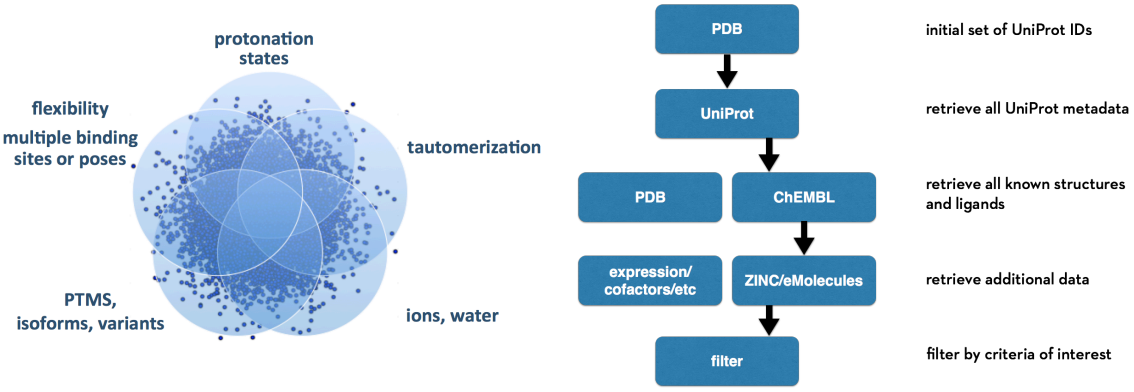

Figure 4. **Mining model protein:ligand systems to focus on individual modeling challenges via a structural and chemical informatics platform.** SAMPL7-10 will feature the introduction of new model protein:ligand systems designed to focus on individual challenges judged to be of critical immediate importance following current D3R/SAMPL blind competitions. *Left:* Since most protein targets of pharmaceutical interest feature a multitude of conflated challenges to quantitative accuracy, our goal is to identify model protein targets that isolate individual effects to focus community efforts by fielding new blind challenges. *Right:* In order to rapidly develop new experimentally- and computationally-tractable model protein:ligand systems, we have developed a structural and chemical informatics system that applies successive filters to the set of *all* potential protein:ligand systems for which structural data is available. [JDC: This figure is a placeholder.]

Aim 4. Coordinate, run, and analyze blind challenges to advance modeling of binding. The data collected in Aims 1-3 will drive annual SAMPL blind challenges, allowing the field to test the latest methods and force fields to assess progress, compare them against one another head-to-head, and perform sensitivity analysis to learn how much different factors (protonation state, tautomer selection, solvent model, force field, sampling method, etc.) affect predictive power. Results will then feed back into improved treatment of these factors for subsequent challenges, driving regular cycles of application, learning, and advancement.

TIMELINE

COLLABORATION MANAGEMENT PLAN

OUTLOOK

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